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Concise Review

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Using Pluripotent Stem Cells to Understand Normal and Leukemic Hematopoietic Development

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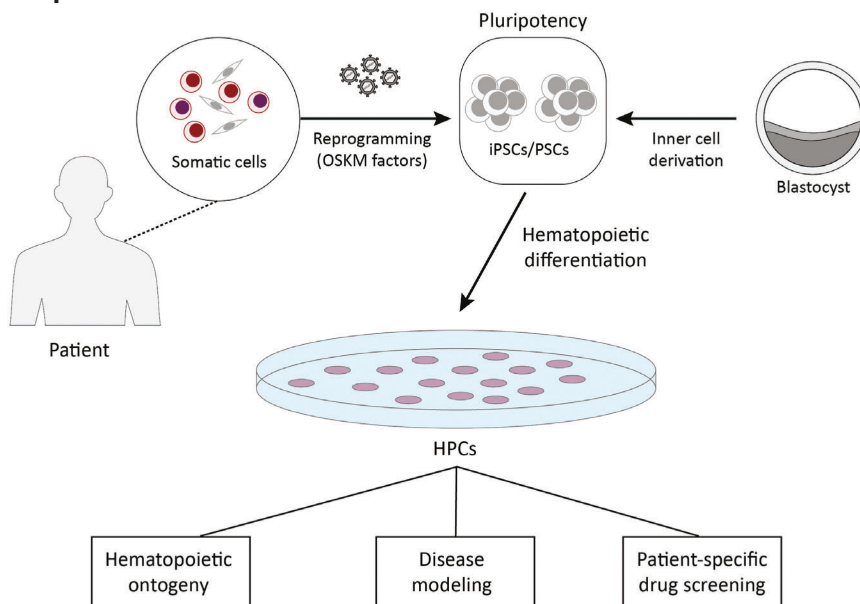
Abstract

Several decades have passed since the generation of the first embryonic stem cell (ESC) lines both in mice and in humans. Since then, stem cell biologists have tried to understand their potential biological and clinical uses for their implementation in regenerative medicine. The hematopoietic field was a pioneer in establishing the potential use for the development of blood cell products and clinical applications; however, early expectations have been truncated by the difficulty in generating bonafide hematopoietic stem cells (HSCs). Despite some progress in understanding the origin of HSCs during embryonic development, the reproduction of this process in vitro is still not possible, but the knowledge acquired in the embryo is slowly being implemented for mouse and human pluripotent stem cells (PSCs). In contrast, ESC-derived hematopoietic cells may recapitulate some leukemic transformation processes when exposed to oncogenic drivers. This would be especially useful to model prenatal leukemia development or other leukemia-predisposing syndromes, which are difficult to study. In this review, we will review the state of the art of the use of PSCs as a model for hematopoietic and leukemia development.

Key words: pluripotent stem cells; adult haematopoietic stem cells; embryo; embryonic stem cells (ESCs); hematologic malignancies.

Graphical Abstract



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Significance Statement

Using pluripotent stem cells for studying the hematopoietic system still holds great potential in terms of generating blood cell types and in modeling leukemia. There has been some constant progress in the field for more than 4 decades but still many challenges need to be overcome. We will review the most promising advances and future directions in this field.

Hematopoietic Development: In Vivo and In Vitro

Embryonic Hematopoiesis (In Vivo)

Hematopoietic stem cells (HSCs) give rise to different types of intermediate progenitors that form all blood cell types in adult organisms. However, during embryonic development, the hematopoietic hierarchy is different and HSC-independent hematopoiesis exists (reviewed in Dzierzak and Bigas[1]). In vertebrates, 3 hematopoietic waves take place in the embryo after gastrulation: (1) the first wave occurs in the yolk sac (YS) at embryonic day (E)7.5 in the mouse, in which primitive erythrocytes, macrophages, and megakaryocytes are formed to sustain the initial oxygen requirement and tissue remodeling; (2) the second wave (mouse E8.5-E9.5) takes place in the YS, but also extends to allantois and embryo itself. Erythroid and myeloid progenitors (EMPs) and B-1a cells[2] are formed and will develop into long-lived tissue-resident macrophages and innate-like B cells, respectively. (3) The third wave (mouse E10.5-E11.5) takes place in the Aorta-Gonad-Mesonephros (AGM) region and detection of the first HSCs with in vivo blood regeneration capacity after transplantation occurs. Cells from the second and third waves are generated from a specialized subtype of endothelial cells called hemogenic endothelium (HE). In the midgestation aorta, HE cells undergo endothelial-to-hematopoietic transition (EHT) giving rise to intra-aortic hematopoietic clusters (IAHCs) that contain hematopoietic stem and progenitor cells (HSPCs) with multilineage potential as well as very few bona-fide HSCs (approximately 2 HSCs out of the 700 formed ckit+ cells).[3] Shortly after their formation, HSPCs migrate to the fetal liver (FL), where they actively self-renew and acquire definitive/adult HSC features. Finally, in mammals HSCs migrate to the bone marrow, where they persist during the lifetime of the organism.

In the human embryo, hematopoiesis also follows a similar multi-site progression but covers a longer time period. The important events begin also in the YS, around Carnegie Stages (CS) 7-8 corresponding to 16-18.5 days post-coitum (dpc), and continue in the AGM from CS13-17 to finally seed the fetal liver. Human YS is a balloon-like structure that produces primitive, nucleated erythrocytes, megakaryocytes, and macrophages.[4] After the establishment of heartbeat in CS10 (21-22 dpc), IAHCs are observed between CS13-17 exclusively in the ventral wall of the dorsal aorta and HSCs can be detected.[5,6] When EHT begins (CS13), hematopoietic cells start also seeding the FL and their proliferation and maturation in this niche extends until birth.[4] The bone marrow niche is established only around CS 23 (56 dpc) and is first homed by CD34-CD45+ hematopoietic cells, mostly comprising of CD68+ monocyte/macrophage population, followed by the colonization of CD34+CD45+ HSPCs.[4] Intriguingly, even though only very few (1 or 2) HSCs are found in the AGM region of both murine and human embryos, the regenerative potential of these HSCs in humans is extremely high, as suggested by transplantation experiments into immunodeficient mice.[6] Moreover, compared to the murine counterpart, a pro-definitive wave has not yet been characterized in human embryos,[4] but likely due to the inaccessibility and unavailability of these samples.

HSCs are the source of transplantation for different therapeutic indications in which the hematopoietic system needs to be replaced. Due to the clinical relevance of these applications, and the lack of HLA compatible donors for some patients, there is great interest in the generation of HSCs in vitro. Understanding the events that guide HSC specification is crucial, not only for regenerative medicine purposes but also for a better understanding of leukemic transformation. In this sense, it is important to dissect the signaling pathways involved in HSC specification and how their dysregulation can lead to the development of different types of leukemia.

Pathways Involved in HSC Formation

Different signaling pathways are known as regulators of the EHT process in the ventral wall of the dorsal aorta (reviewed in Perrimon et al[7]). In this section, we briefly describe the main pathways involved in the generation of HSPCs/HSCs regulating the activation of the heptad transcription factors (SCL, LYL1, LMO2, GATA2, RUNX1, ERG, and FLI-1), required for the HSC program.[8]

Notch Signaling Pathway

Notch is a cell-to-cell contact signaling pathway that is required for HSC specification within the AGM region (reviewed in Bigas and Porcheri[9]). It comprises different receptors (NOTCH1-4) and ligands (JAG1, JAG2, DLL1, DLL3, and DLL4), although not all of them are expressed in the AGM region nor have key functions in the hematopoietic onset. In more detail, Jag1/Notch1 activity directly regulates, through an "incoherent feed-forward" loop, the expression of Gata2 that, in turn, positively impacts the Runx1 expression.[10] Notch signaling has to be very tightly regulated in the AGM region, since it is required for both hematopoietic and arterial fates, and differences in the output (hematopoietic or arterial) seem to be linked to threshold levels of Notch activity.[11]

Wnt/β-Catenin Pathway

Wnt/β-catenin is highly conserved among species and it controls cell fate and embryonic patterning across evolution. There are different Wnt ligands, Frizzled receptors, and LRP coreceptors (reviewed in Angers and Moon[12]), and the main effector of the canonical Wnt signaling is the β-catenin transcription factor. Pioneering work carried out in zebrafish[13] and mouse[14]demonstrated the importance of β-catenin in HSC generation. Deletion of β-catenin in embryonic endothelial cells or pharmacological inhibition of the pathway results in the abrogation of HSC generation, but has not much effect thereafter. The authors proposed a developmental window (around E10.5) in which HSC generation depends on β-catenin activity and gradually this process becomes

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β-catenin independent. β-catenin is also necessary for a proper arterialization through a crosstalk with the Notch pathway by activating Dll4 expression, which is key for arterial specification.[15]

Retinoic Acid

Retinoic acid (RA) is a metabolite of retinol (Vitamin A) that acts as a ligand for nuclear RA receptors (RARs). RA-RAR complex can function as a transcriptional activator or repressor to properly regulate the development of organs and tissues in chordate animals.[16] Its main role in hematopoietic development is the induction of the dormancy state in adult HSCs.[17] In the embryo, there is an expression of different RARs in the dorsal aorta at E11.5 and, upon activation of the pathway in AGM explants, HSC activity was enhanced through a transient downregulation of Wnt signaling.[18] Additionally, RA has been recently used to highlight the heterogeneity of HE populations formed from pluripotent stem cells (PSCs)[19] and in vitro studies based on differentiation experiments suggest a positive impact of RA in hematopoietic progenitor commitment.[20]

BMP Signaling

Bone morphogenetic proteins (BMPs) and Nodal are members of the transforming growth factor-β (TGF-β) family. BMP2B and BMP4 induce the expression of caudal-type homeobox (CDX) genes which, in turn, regulate homeobox (HOX) gene expression required for hematopoietic fate acquisition.[21] The presence of BMP4 in the ventral mesenchyme of the dorsal aorta enhances the HSC production with repopulation activity.[22] In contrast, the BMP inhibitor BMPER impacts positively in the HSC maturation in vitro, thus indicating that there may be different needs for BMPs during HSC development.[23]

Hedgehog Pathway

Hedgehog (Hh) acts as a morphogen to ensure a proper development of many tissues.[7] It is mainly active in the mesenchymal tissue surrounding the ventral wall of the dorsal aorta and is important for the hematopoietic differentiation of ESCs.[24,25] Upon culturing AGM explants with 2 different hedgehog ligands (Indian and Sonic), an enhancement of the HSC activity was observed. Hh-VEGF signaling axis regulates HSC activity culminating in the activation of the Notch pathway.[26]

Inflammatory Signals

Tissue injury or pathogen infection triggers inflammatory pathways that converge in the activation of the NF-κB or interferon (IFN) pathways. It was a remarkable finding that these signals were required for HSC specification both in zebrafish[27] and in the mouse embryo.[28] IFN pathway types I and II, together with TNFα, induce an increase in Ly6a/Sca-1 population within the AGM, which labels definitive HSCs in the dorsal aorta.[28] More recently, an unbiased screen identified TLR4-MyD88-NF-κB axis to be required for HSC emergence in zebrafish and in the mouse embryo,[29] also connected with the Notch pathway.[27] The origin of the inflammatory cues in the sterile environment of the AGM is intriguing. Inflammatory signals may originate from primitive myeloid cells derived from earlier waves of hematopoiesis.[1,30]

In summary, many signals are important in EHT and HSC specification; however, they all need to be tightly regulated and coordinated to produce an HSC, possibly creating a very fine-tuned process that robustly generates a bona-fide HSC. Nevertheless, how the interplay between different pathways controls HSC specification remains undetermined.

Hematopoietic Development in a Dish

The maintenance of the hematopoietic system depends on self-renewing HSCs. In this regard, transplantation of HSCs has become the gold standard of treatment for a wide range of blood diseases. However, the availability of an HLA-compatible HSC donor is rare in some ethnic groups,[31] and even if haploidentical donor transplants are now widely used, alternative sources of HSCs would allow the treatment of more patients. HSCs could be derived from banked cells with a matched HLA repertoire or from patient-derived reprogrammed cells.

Here we summarize the experimental strategies that have been used to generate blood progenitors and the main problems encountered in obtaining bona-fide HSCs for a robust hematopoietic generation.

Pluripotent Stem Cells and induced Pluripotent Stem Cells

Embryonic stem cells (ESCs) are PSC lines derived from the inner cell mass of murine[32] or human[33] blastocysts. They are cultured under specific conditions that preserve their self-renewal capacity.[34]

A non-embryonic source of PSCs can be obtained by reprogramming somatic cells by introducing specific transcription factors (Sox2, Oct4, myc, klf4) that induce pluripotency.[35] Once reprogrammed, the induced PSC (iPSC) remains pluripotent with almost identical features as PSC. Both types of PSCs can be induced to differentiate toward any cell type under specific conditions (reviewed in[36]). The pluripotency or the ability to differentiate into different lineages can be assessed in vivo by teratoma formation by injecting PSCs into murine recipients, or in vitro by defining the right cues (cytokines or small molecules) that activate different developmental pathways and generate "a la carte" cell types.

For hematopoietic differentiation, 2-dimensional (2D) cultures and 3-dimensional (3D) structure protocols have been successfully used by investigators to generate different types of HSPCs and mature cell types from PSCs. The 2D culture is based on the direct contact of PSCs with hematopoietic supportive stromal cells. The most widely used cells are bone marrow derived-OP9 stromal cells.[37] In a first step, PSCs are directed towards mesoderm to later differentiate into multilineage hematopoietic progenitors.[36] In these conditions, a bias toward myeloid and erythroid lineages and very scarce lymphopoiesis has been detected (few B cells with limited functionality).[38] T lymphopoietic potential has also been detected by using OP9 stromal cells engineered to ectopically express Notch ligand Delta-like 1 (OP9-DLL1). In these co-culture conditions, ESCs give rise to CD4+CD8+ T cells.[39]

The 3D structures that are able to produce hematopoietic cells can be formed in vivo by teratoma induction or in vitro as a result of PSC aggregates in non-adherent conditions. The main goal for these models is to better recapitulate the cell–cell interactions that occur in the niche of HSC generation in the embryo.

Teratomas

PSCs self-aggregate and differentiate spontaneously when subcutaneously injected into a mouse and give rise to a benign

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mass (teratomas) containing differentiated cell types from the 3 germ layers (ectoderm, mesoderm, and endoderm).[40,41] While pluripotency of murine ESC can be tested by generating mouse chimeras that contribute to the germline, the teratoma assay is the only possibility to test the pluripotency of human PSC in vivo. PSC-derived teratomas are capable of generating hematopoietic progenitors. In this regard, 2 independent studies demonstrated that in vivo teratoma formation from human iPSCs can lead to the formation of a small fraction of HSC-like cells.[42,43] In both cases, the reconstitution capacity was very low, but they demonstrated that HSC-like cells can be generated from PSCs. Interestingly, co-injection of iPSC with stromal cells increased the multilineage repopulating activity suggesting that recreating a 3D niche might favor the generation of HSCs.[43]

Embryoid Bodies

The self-aggregation capacity of PSCs was first recapitulated in vitro when teratocarcinoma cells were cultured in the absence of a feeder layer and produced embryoid bodies (EBs) that are 3D structures resembling early embryos, constituting multipotent and differentiated cells from all 3 germ layers (ectoderm, mesoderm, and endoderm).[44,45] EBs derived from human or murine ESCs have been used to study cell fate decisions and the differentiation of various cell types during embryogenesis as a good model for hematopoietic development.[46,47]

For years, investigators have tried to mimic the in vivo development of HSPCs from HE and mesodermal precursors by incubation with specific factors. The majority of conditions lead to the generation of hematopoietic cells that resemble the primitive wave, although definitive hematopoietic cells when cultured under specific conditions have been obtained.[48-50]

Current knowledge supports that primitive and definitive hematopoietic waves progress through an HE intermediate in contrast to a previous notion that primitive hematopoiesis arose from bipotent hemangioblasts.[51]

Over the last 2 decades efforts have been made to define the right condition to obtain definitive HSCs capable of long-term repopulation (see Fig. 1). Several groups using the EB-based system identified some of the signaling pathways involved such as Wnt or TGFβ/BMP-4, as well as the key function of the developmental regulators HOX genes in definitive hematopoietic commitment.[50,52,53]

There are several examples of successful induction of phenotypic HSCs from murine or human PSCs.[42,43,54-56] Overexpression of HoxB4 in mouse ESC-derived hematopoietic lineages co-cultured on OP9 cells generated functional progenitors after transplantation with adult-type erythroid, myeloid and lymphoid lineages, indicating a successful generation of HSCs.[54] However, these results could not be reproduced in human PSC-derived blood.[57] In human PSCs (hPSCs), different temporal requirements of growth factors such as BMP4, Activin, bFGF, and VEGF[58] or Wnt activation by CHIRON[48]have been defined to obtain hemogenic/hematopoietic cells that are capable of multilineage repopulation potential, although they lack the ability for long term engraftment.

Combining differentiation with genetic induction, myeloid precursors derived from hPSCs were induced for HSC specification (by introducing HOXA9, ERG, RORA, SOX4, and MYB genes). This combinatorial approach allowed the generation of multipotent progenitors with erythroid and myeloid potential, also displaying the ability to short-engraft into NSG mice.[55] Four years later, this group reported the generation of bona-fide HSCs after redirecting hPSC-derived hemogenic endothelial cells by the activation of 7 transcription factors (TFs) (*ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, SPI1*). Generated cells exhibited long-term engraftment and self-renewal potential (validated by secondary transplantation experiments) as well as differentiation into all the adult blood lineages, resembling totally functional HSCs.[56]

The generation of functional adult-like differentiated blood cell types has been more successful. Current protocols exist for generating macrophages, erythrocytes, natural killer or T cells from EBs useful for clinical applications.[59-61]

In summary, most culture conditions recapitulate the pro-definitive wave with limited multilineage and engraftment potential. Few successful cases are not easily reproduced and require genetic intervention which is not useful for clinical applications.

Organoids and Gastruloids as a Tool to Modulate Hematopoietic Development In Vitro

Organoids are a 3D tissue-specialized in vitro culture to generate structures of self-organized tissues that can resemble organs. They display a higher level of tissue organization than tissues formed in EBs.[62] They assemble the niche-like microenvironment that creates and sustains tissue stem cells over time (eg, intestinal and skin organoids). This ability to self-organize and resemble particular in vivo organs led to the hypothesis that hematopoietic organoids might reproduce the events associated with early hematopoiesis in the embryo and HSC generation. In this sense, Elefanty et al[53] developed a combined method in which PSCs were initially polarized toward hemato-vascular mesoderm via EB culture and when transferred to adherent culture conditions, gave rise to a network of vascular structures from which hematopoietic cells bud off. This structure resembled the niche in the AGM region (AGM-like hematopoietic organoids) producing progenitors with a transient output of myeloid cells and later erythroid cells. More recently, using a RAG1:GFP reporter labeling lymphoid progenitors, they identified a subset of RAG1+ hematopoietic precursors with multipotent potential (erythroid, myeloid, and lymphoid) that originated from vascular cells and express genes typically associated to blood progenitors.[63] Nevertheless, these hematopoietic organoids lack in vivo engraftment potential and the organoid-generated blood cells had to be further exposed to stromal cells to differentiate into mature cells, suggesting the absence of appropriate environmental cues in the organoids.

An improved 3D culture system is the so-called gastruloids. Gastruloids are embryonic organoids resulting from the aggregation of precise numbers of PSCs that, under defined culture conditions, develop an organization that mimics the early mammalian embryo.[64,65] After 7 days in culture, gastruloids from mouse PSCs develop a complex spatial organization with derivatives of the 3 germ layers arranged with respect to 3 orthogonal axes in a manner that mirrors the organization of the mouse embryo at E9.5. Gastruloids lack a brain and extraembryonic tissues but contain progenitors of the other organs and tissues of the body. Modifications of the original protocol can bias the outcome toward specific embryonic structures, eg, cardiac primordium[66] or somites[67] and spinal cord.[68] A by-product of the "cardiac gastruloids" is the presence of endothelial cells with properties of hemogenic

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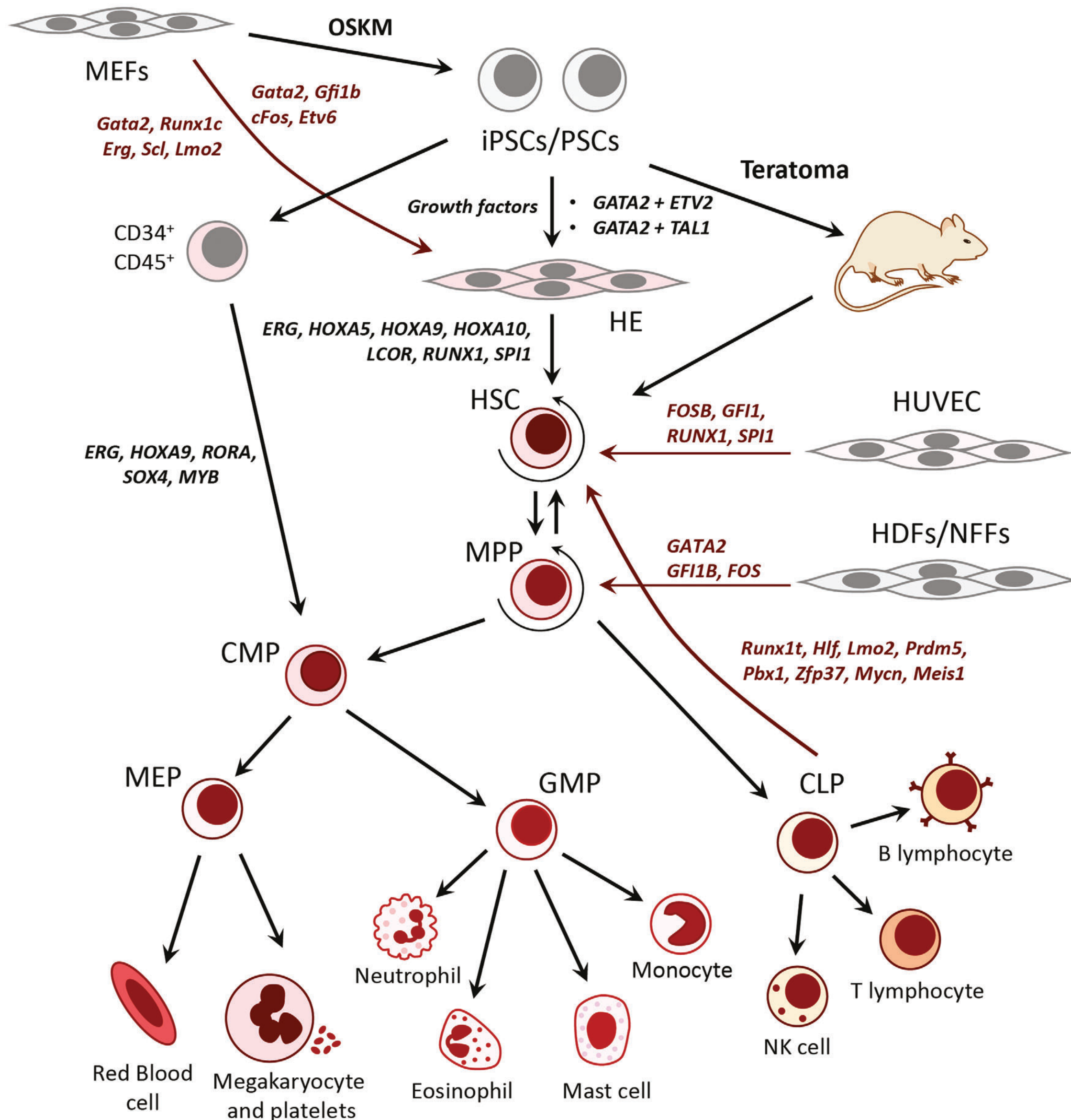

Figure 1. Multiple strategies to generate HSCs in vitro. Summary of strategies used for HSC formation from different cell sources. Embryonic stem cells [ESCs], and induced pluripotent stem cells [iPSCs] are used for direct differentiation, fibroblasts for direct reprogramming to iHSC, lineage committed blood progenitors for de-differentiation, and endothelial cells for transdifferentiation. Specific combinations of transcription factors have been used in each situation. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; HE, hemogenic endothelium; GMP, granulocyte-macrophage progenitors; HSC, hematopoietic stem cell; HUVEC, human umbilical vein endothelial cell; MEP, megakaryocyte-erythrocyte progenitor; MPP, multipotent progenitor; OSKM, Oct4, Sox2, Klf2 and Myc (Yamanaka's factors); MEFs, mouse embryonic fibroblasts; HDFs, human dermal fibroblasts; NFFs, neonatal foreskin fibroblasts. Arrows denote reprogramming/dedifferentiation approaches with capital letters refering to human genes and small letters refering to murine counterparts.

progenitors supporting the potential use for hematopoietic development.[69]

Reprogramming Somatic Cells Toward Blood Progenitors and HSC-Like States

Somatic cell types have also been used to generate hematopoietic progenitor cells (HPCs) through TFs-mediated reprogramming (see Fig. 1), but the first attempts were performed by fusing human embryonic fibroblast with fetal liver cells.[70] Reprogrammed progenitors showed multilineage potential but not in vivo engraftment. More recently, a minimal combination of 4 TFs (Gata2, Gfi1b, cFos, and Etv6) allowed a direct conversion of mouse fibroblast toward blood progenitors with myeloid, erythroid, and megakaryocytic potential across EHT.[71] These findings have been translated into human cells by reprogramming human dermal and neonatal fibroblasts into lymphoid and myeloid progenitors with limited repopulation capacity.[72] Using a similar approach, murine fibroblasts were successfully reprogrammed toward hematopoietic progenitors with short-term engraftment capacity by the forced expression of 5 TFs (Gata2, Erg, Tal1, Runx1, and Lmo2) and expansion on stromal cells.[73]

Similarly, human umbilical vein endothelial cells (HUVEC) were converted by enforced expression of FOSB, GFI1, RUNX1, and SPI1to functional multipotent progenitor cells (MPPs) with the ability to give rise to not only myeloid output but also NK cells and B lymphocytes,

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engrafting in primary and secondary immunocompromised murine recipients.[74]To improve this protocol, adult mouse endothelial cells with transient expression of these factors were co-cultured with an inductive vascular niche. In these conditions, endothelial cells were reprogrammed toward an HSC-like state with clonal self-renewal capacity and multipotent reconstitution potential. Both myeloid and lymphoid lineages with ability to induce adaptive immune responses were generated.[75]

Murine pro-B cells were also reprogrammed by the transient forced expression of 6 TFs (Pbx1, Prdm5, Hlf, Lmo2, Runx1, and Zfp37) into HSC-like cells. These induced HSCs (iHSCs) demonstrated the capability to serially reconstitute the HSPC compartment in recipient mice, with myeloid, erythroid, megakaryocytic, and lymphocytic (both T and B) lineages.[76]

Malignant Hematopoiesis: Leukemia Development and How it can be Modeled in vitro Through the Use of PSCs

Modeling human leukemia is a great challenge that can improve patient therapeutic strategies. Several pediatric leukemias have been shown to initiate in utero and their biology needs to be understood in the context of embryonic development. Among them, the most aggressive is infant leukemia, defined as those that affect patients during the first year of life. The most common type is B cell acute lymphoblastic leukemia (B-ALL) in which the first hit is usually a translocation resulting in fusion proteins such as MLL-AF4, MLL-AF9, or TEL-AML1.[77] Acute megakaryoblastic leukemia (AMKL)[78] also has a developmental origin, occurring with high frequency in Down syndrome (DS) patients, but also in non-DS patients linked to chromosomal translocations. Many point mutations, such as those occurring in the Notch1 gene, a T-cell lymphoblastic leukemia (T-ALL) driver, have also been detected in blood spot analysis at birth.[79]

Elegant studies in monozygotic twins demonstrated that the first hit in infant leukemia occurred in utero and resulted in a pre-leukemic clone existing in both twins, but not necessarily developing into a leukemic clone in both of them.[80] However, to study the effect of such mutations and the evolution of pre-leukemic clones during human embryonic development in vivo is not possible and mouse models are not yet able to faithfully reproduce a similar disease.[81,82]

In this context, PSCs give a unique opportunity to recapitulate some of the human embryonic events in vitro in the presence of first leukemic hits since they have to progressively go through different embryonic developmental stages to become hematopoietic cells. Moreover, the ability to obtain genetically modified PSC or patient-derived iPSC opens a window of opportunity to test many genetic conditions as it happens with other genetic diseases. Nevertheless, the main obstacles to modeling these events from PSCs remain the limited output of blood progenitors,[83] as well as the nature of generated HPCs, which are mainly myeloid, thus limiting the modeling of lymphocytic neoplasms.

In recent years, several leukemic models have been engineered in PSCs with the capacity to reproduce leukemia in transplanted mice, thus opening a window of opportunity for the use of these models in leukemia research (see Fig. 2). In the following sections, we will review some of these successful models.

Modeling Myeloid Malignancies

The first PSC-based model for leukemia was engineered by introducing the BCR/ABL oncoprotein, a common lesion found in myeloid leukemias, into mouse ES cells.[84] Authors observed an expansion of undifferentiated progenitors with a change in the balance of the myeloid-erythroid output, which generated promising expectations for the use of these models. After the development of iPSC technology, several groups have modeled myeloid neoplasms by reprogramming patient-derived blood cells, providing a faithful model to study genetic abnormalities that are present in patients (reviewed in Papapetrou[85]). In 2010, Carette et al were able to reprogram KBM7 Chronic myeloid leukemia (CML)-derived cells that carry the BCR/ABL translocation.[86] Interestingly, these reprogrammed pluripotent cells failed to respond to a tyrosine kinase inhibitor, imatinib, although sensitivity to the treatment was required after hematopoietic differentiation. Despite some success in CML reprogramming, it was early found that most leukemic cells were harder to reprogram than normal cells, and the process was selected for those clones with less or no mutations. This feature has been crucial to understanding leukemia clonal evolution in iPSC (developed in next section). Another interesting feature of reprogramming is that the epigenetic landscape is mainly reset in iPSC[87,88]; however, leukemia-associated epigenetic changes might be recapitulated after differentiation, as has been shown for CG-DNA methylated regions.[87]

Other iPSCs-based myeloid malignancies models have been established by engineering recurrent chromosomal deletions found in patients into healthy iPSCs by CRISPR-Cas9 technology.[89] This approach has allowed the authors to identify novel genes involved in myelodysplastic syndromes (MDS) that were implicated in the pathogenesis and could be targetable for therapeutic purposes. In addition, some hotspot mutations, such as JAK2 V617F, MPL, and *CALR* have been identified in patient-derived iPSCs,[85] although disease-defining criterion has been mainly the formation of colonies in methylcellulose independent of cytokines, no engraftment potential of iPSCs-derived leukemic hematopoietic progenitors was described.

Leukemia Evolution

iPSCs can be used to model the transition between different stages of the disease. In some AML patients, normal cells or cells with less genetic lesions seem to have a higher capacity to be reprogrammed than fully leukemic cells.[90] Different selection strategies have been designed to be able to capture the more difficult leukemic clones. In this regard, a panel of iPSC lines containing different lesions was generated and allowed to capture from pre-leukemia to acute myeloid leukemia (AML) through the MDS stage.[90] Correction of the progression-associated mutation by CRISPR-Cas9 allowed reversion of the disease. Another intriguing result was that AML-iPSC-derived hematopoietic cells have the ability to robustly engraft into NSG mice after intravenous injection without any gene modification, and just by a directed differentiation. Similar results were described for MLL-translocated AML-iPSCs that gave rise to an aggressive myeloid leukemia in vivo.[91] Recently, Papapetrou's group was able to not only capture the clonal evolution of AML but also to identify early genetic events that lead to disease onset and can be clinically relevant to stop disease progression.[92]

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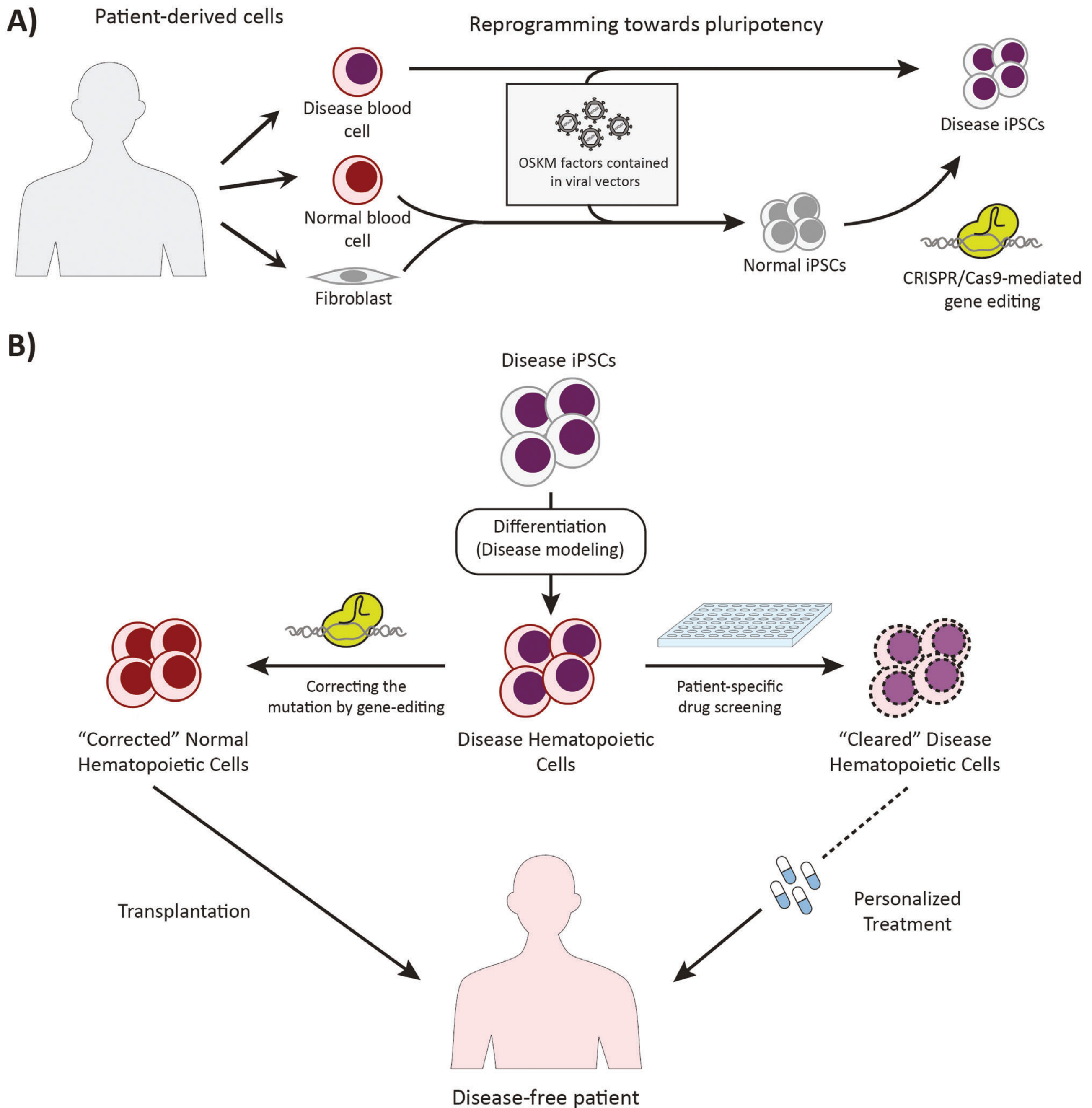

Figure 2. Different strategies of iPSCs use in blood disease modeling and personalized medicine. (**A**) Patient-derived somatic cell types (fibroblast and pathological or non-pathological blood cells) can be reprogrammed toward iPSCs. Patient-derived iPSCs harbor genetic lesions involved in disease pathogenesis. (**B**) Patient-derived iPSCs or CRISPR/Cas9-engineered iPSCs can be differentiated into the appropriate cell-type, fully or partially recapitulating the pathogenesis in vitro, to uncover mechanisms of drug resistance or select specific response drugs for each patient. A promising approach for monogenetic syndromes consists in correcting the mutated gene by gene-editing technology and then transplant them back into the patient.

Modeling Genetic Predisposition to Leukemia

Certain mutations that are inherited or occurred de novo in the germline can also lead to hematopoietic malignancies. Such mutations, either directly in hematopoiesis-specific genes like in RUNX1, CEBPA, ETV6, GATA2 or associated with other inherited chromosomal aberrations, like inherited bone marrow failure syndromes, neurofibromatosis 1, Ataxia telangiectasia (AT) predisposes the individual to cancer, contributing to a significant proportion of childhood leukemias.[93] The malignant transformation might depend on the type of mutation and the somatic/epigenetic alterations that get accumulated further. Proper modeling of these malignancies to underpin the specific genetic aberration that leads to this predisposition will facilitate the prevention or treatment of the cancers.

Juvenile myelomonocytic leukemia (JMML) is an aggressive and fatal, pediatric myeloproliferative disorder caused by the somatic and/or germline mutations in signaling genes, like PTPN11, CBL, NRAS, KRAS, etc.[94] It is associated with constitutive activation of GM-CSF signaling, along with

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other pathways like RAS/MAPK, JAK/STAT and PI3K/Akt/mTOR.[95] Patient-derived iPSCs (from peripheral blood or non-hematopoietic cells like fibroblasts) harboring somatic/inherited *PTPN11* mutation when induced for hematopoiesis showed a bias to myeloid lineage, higher proliferation, and constitutive activation of GM-CSF, thus perfectly modeling the disease.[95,96] More importantly, the in vitro iPSC model has been successful in distinguishing the different signaling pathways hyperactivated in response to distinct germline mutations and testing specific drugs that inhibit the pathways, opening the possibilities for efficient treatment of this leukemia.[95]

GATA2 deficiency is a complex multi-system disorder, caused by germline heterozygous mutation in *GATA2* and characterized by bone marrow failure, immunodeficiency, and high risk of developing MDS and AML.[97] Penetrance and expressivity within GATA2 families are often variable, suggesting that cooperating somatic mutations and/or epigenetic events are required to trigger the disease. Nowadays, chemotherapy and allogeneic HSC transplantation are the only curative options. A first attempt to model GATA2 deficiency by patient-derived iPSCs has been recently described.[97] However, hematopoietic differentiation of these patient-derived iPSCs did not recapitulate the monocytopenia observed in the GATA2 carriers.

The absence of significant defects in iPSC hematopoietic development might be due to the primitive rather than definitive state of the hematopoietic progenitor cells, the lack of external stress factors, the absence of secondary drivers, and/or different epigenetic regulation of mutant vs. wildtype GATA2 allele.[97] Most recently, Castaño et al. used CRISPR-mediated gene editing to introduce in a healthy hiPSC line 2 of the most recurrent germline *GATA2* mutations associated with MDS.[98] The generation of a de novo GATA2 deficiency disease model in a normal genetic background might help to better understand the role of GATA2 haploinsufficiency on hematopoietic development.

Down syndrome (Trisomy 21) patients, suffering from serious physical and cognitive defects, are also at risk of myeloid and lymphoid leukemias, with an estimated 500-fold increased risk of acute megakaryocytic leukemia (AMKL) in children.[99] Leukemogenesis occurs in a stepwise manner, beginning at the FL hematopoiesis, with an uncontrolled expansion of megakaryocytes, incomplete differentiation of erythroid cells, and a differential block to B cells regulated by a truncated version of GATA1 (GATA1s) that is observed in all DS patients, formed by multiple mutations in *GATA1*.[99] Successful modeling of the aberrant hematopoiesis in DS patients was done in isogenic iPSC lines that differed only in the number of chromosome 21 copies (disomic cell lines considered as control), where robust colony-expansion of erythroid, myeloid and megakaryocytic lineage cells are observed at a developmental stage corresponding to FL hematopoiesis, identified based on γ-globin expression.[99]

Modeling of Lymphoid Malignancies

The T and B lymphocytes are vulnerable to malignant transformations due to the multiple genetic rearrangements and clonal expansion that is required for their maturation and function. B-cell acute lymphoblastic leukemia (B-ALL) is characterized by extensive proliferation of lymphoblasts and a differentiation block toward mature B cells, initiated by chromosomal translocations like MLL-AF4 and ETV6-RUNX1 in utero that produces pre-leukemic cells followed by the accumulation of supporting mutations triggering the leukemia,[77,80].The lack of accessibility of patient samples at the pre-leukemic stage, the tendency of acute leukemic cells to undergo apoptosis in culture, and the distinct physiology of mouse models make it extremely difficult to study the occurrence of the malignancy. Although some progress has recently been made by inducing MLL rearrangements in fetal liver cells and letting them develop until adulthood,[82,100] obtaining a reliable model is still a challenge. Importantly, the in vitro development of HSPCs from ESCs does not efficiently model lymphoid development and the B-ALL cells are resistant to reprogramming to induced pluripotent cells.[101] Still, investigators are trying to develop a reliable system, for example, using lentiviral transduction of hESCs to study the co-operation of the reciprocal fusion genes MLL-AF4 and AF4-MLL, both of which, individually and in combination, are known to trigger a very aggressive form of infant pro-B-ALL.[102] They observed that the cooperation of the fusion genes leads to an improved differentiation to hemato-endothelial progenitors (2-3 fold) and thereby an increase in HSPC numbers. Although this may be biologically important, it does not recapitulate the leukemic transformation in the HSPC cells.

The t(12;21) chromosomal translocation giving rise to ETV6-RUNX1 (TEL-AML1) fusion gene, which represses RUNX1 transcription, is the most common aberration causing infant B-ALL.[103] Studies using murine HSPC cell line EML1 (inducible to myeloid, erythroid, or lymphoid lineages using specific cytokines) transduced with the ETV6-RUNX1 fusion gene showed inhibition of the IFNα/β signaling leading to B cell differentiation block; the result was validated in a patient-derived, B-lymphocytic leukemia cell line, REH, by using shRNA to silence the fusion gene.[103] Most importantly, a detailed and elegant study characterized a developmental stage-restricted, transient B-cell progenitor population during human embryonic hematopoiesis that has lympho-myeloid capacity.[104] The population, identified as IL-7R+CD19- and expressing RAG, goes from a predominantly immune-biased myeloid state (capable of producing macrophages but not erythrocytes) to a lymphoid state from the developmental Carnegie stage (CS) 17 to CS20. The ETV6-RUNX1 translocation is identified to occur in this population and is speculated to dysregulate the transition from myeloid to lymphoid state, indicated by the presence of few myeloid cells expressing RAG and the fusion gene.[104] Interestingly, differentiation of hPSCs to B cells by co-culture with OP9/MS5 stromal cell line recapitulated this transition in vitro, and homologous recombination knockin of ETV6-RUNX1 into the hPSCs prior to co-culture led to differentiation block as seen in the embryos, perfectly modeling the first hit in the leukemic transformation.

Remarks and Future Goals

PSCs are still the most promising cell source for generating ex vivo HSCs. Although many efforts have been dedicated to HSC development, and some promising proof-of-concept results have been published, we are still unable to reproducibly generate de novo HSCs in vitro. Our knowledge in embryonic hematopoietic development in model organisms, but also in the human embryo, is now opening new avenues

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to explore in these cellular systems. New technologies such as microfluidics and 3D cultures, relying on self-organization of the PSC will provide new strategies to address this problem.

Interestingly, using PSCs for leukemia modeling has been more rewarding than generating HSCs. PSC-derived hematopoietic cells have been transformed into myeloid leukemic cells. In some cases, engraftment capacity in immunocompromised mice has been achieved and recapitulated some steps of leukemic transformation. Although these models are not as robust as patient xenograft-derived models or genetic murine models, they are especially attractive for leukemia-predisposing syndromes or pre-natally occurring leukemia. In the latter, the mutations occur or are present during the embryonic time when developmental signals are active and influence the evolution of pre-malignant populations. Reproducing the embryonic niche where these cells are generated by differentiating PSCs may provide additional cues to understand the evolution and penetrance of these diseases. Another important advantage of PSCs in leukemia modeling is the fact that rare cellular pre-leukemic clones can be captured by reprogramming due to the advantage that normal cells have in this system. The availability of multiple reprogrammed leukemic clones can provide important information about the clones observed. Still modeling lymphoid malignancies is a challenge, and the success will likely rely on reproducing a faithful embryonic hematopoietic development in vitro.

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Conflict of Interest

The authors declared no potential conflicts of interest.

Author Contributions

A.B., G.M.K.: conceptualization, manuscript writing, final review. L.G.P.: conceptualization, formal analysis, manuscript writing, final review. A.G.: conceptualization, final report, manuscript writing, final review.

Data Availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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